Agriculture and Agri-Food Canada — Agriculture et Agroalimentaire Canada

Plant Products Division
Food Production and Inspection Branch
Agriculture and Agri-Food Canada

Decision Document **DD95-01**

Determination of Environmental Safety of Agrevo Canada Inc.'s Glufosinate Ammonium-Tolerant Canola

This Decision Document has been prepared to explain the regulatory decision reached under the guidelines Dir94-08 Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits and its companion document Dir94-09 The Biology of *Brassica napus* L. (Canola/Rapeseed), and the proposed guidelines Pro94-04 Guidelines for the Assessment of Plants with Novel Traits as Livestock Feed.

The Plant Biotechnology Office and the Feed Section of the Plant Products Division have evaluated information submitted by AgrEvo Canada Inc. regarding a glufosinate ammonium-tolerant and kanamycin-resistant canola line. They have determined that this plant with novel traits does not present altered environmental interactions when compared to currently commercialized canola varieties and is considered substantially equivalent to canola currently approved as livestock feed.

Unconfined release into the environment, including feed use of HCN92, and other *B. napus* lines derived from it, but without the introduction of any other novel trait, is therefore considered safe.

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Plant Products Division
Food Production and Inspection Branch
59 Camelot Drive
Nepean, Ontario
K1A 0Y9
(613) 952-8000
Facsimile: (613) 992-5219

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I. Brief Identification of The Plant With Novel Traits (PNT)

Designation(s) of the PNT:	HCN92
Applicant:	AgrEvo Canada Inc.
Plant Species:	Canola (*Brassica napus* L.)
Novel Traits:	Glufosinate ammonium (herbicide) tolerance; kanamycin (antibiotic) resistance
Trait Introduction Method:	*Agrobacterium tumefaciens*-mediated transformation
Proposed Use of PNT:	Production of *B. napus* for seed oil for human consumption and seed oil and meal for livestock feed. These materials will not be grown outside the normal production area for canola.

II. Background Information

AgrEvo has developed a *Brassica napus* canola line tolerant to glufosinate ammonium, a broad spectrum non-residual herbicide. This *B. napus* line, referred to as HCN92 in the present document, will allow the use of glufosinate ammonium as a post-emergence herbicide, thus providing an alternative for weed control in canola production, and reducing reliance on soil-incorporated herbicides.

The development of HCN92 was based on recombinant DNA technology, by the introduction of two bacterial genes into a line of *B. napus*. A gene conferring tolerance to glufosinate ammonium was inserted, coding for phosphinothricin acetyltransferase, an enzyme that inactivates glufosinate ammonium through acetylation. Another gene, conferring resistance to kanamycin, was also inserted; this gene is of no agronomic interest but was used to select modified plants from those that remained unmodified at the development stage.

HCN92 has been field tested in Canada under confined conditions in Saskatchewan (1990-94), Alberta (1991-94), Manitoba (1991-94), and Ontario (1993-94).

AgrEvo has provided data on the identity of HCN92, a detailed description of the modification method, data and information on the stability of the gene insertion, the role of the inserted genes in donor organisms and the role of regulatory sequences in donor organisms, their molecular characterization, and full nucleotide sequences. The novel proteins were identified and characterized, including their potential toxicity to livestock and non-target organisms, allergenicity, and levels of expression in the plant and feed. Numerous detailed scientific publications were also supplied.

Agronomic characteristics such as seed production, time to maturity, flowering period, and male and female fertility were compared to those of unmodified *B. napus* counterparts. Effects of HCN92 residues on growth and productivity of the following season's grain, forage, and pulse crops were assessed.

AgrEvo has also provided data on HNC92's survival adaptations: silique shattering potential, seed dormancy, seed dispersal mechanisms, vegetative vigor, reproductive characteristics, and the emergence in subsequent years of volunteer plants under mechanical or chemical fallow conditions. Stress adaptation was evaluated, including susceptibilities to various *B. napus* pests and pathogens, to abiotic stresses such as soil salinity and moisture regimes, and to herbicides other than glufosinate ammonium that are normally used on canola crops. Invasiveness studies were performed under disturbed, undisturbed, and agronomic conditions.

Data to support the efficacy of HCN92 as a livestock feed were provided. A proximate analysis to include crude protein, crude fat, crude fiber, ash and gross energy were supplied for the whole seed, processed meal and oil content.

Agriculture and Agri-Food Canada (AAFC) has reviewed the above information, in light of the assessment criteria for determining environmental safety of plants with novel traits, as described in the regulatory directive Dir94-08:

- potential of the PNT to become a weed of agriculture or to be invasive of natural habitats,
- potential for gene-flow to wild relatives whose hybrid offspring may become more weedy or more invasive,
- potential for the PNT to become a plant pest,
- potential impact of the PNT or its gene products on non-target species, including humans, and
- potential impact on biodiversity.

AAFC has also reviewed the above information in light of the assessment criteria for determining safety and efficacy of livestock feed, as described in Pro94-04:

- potential impact on livestock, and
- potential impact on livestock nutrition.

III. Description of the Novel Traits

1. Glufosinate Ammonium Tolerance:

- Phosphinothricin (PPT), the active ingredient of glufosinate ammonium, inhibits glutamine synthetase, which results in the accumulation of lethal levels of ammonium in susceptible plants within hours of application.

- The phosphinothricin tolerance gene engineered into HCN92 codes for PPT-acetyltransferase (PAT). This enzyme detoxifies phosphinothricin by acetylation into an inactive compound. It has extremely high substrate specificity; experimental data clearly showed that neither L-PPT's analog L-glutamic acid, D-PPT, nor any protein amino acid can be acetylated by the PAT enzyme.

- The PAT gene was originally isolated from *Streptomyces viridochromogenes*, an aerobic soil actinomycete. The PAT enzyme is therefore naturally occurring in the soil. More generally, acetyltransferases are ubiquitous in nature.

- The gene is linked to a constitutive promoter, and protein expression was detected in roots, leaves, buds and seeds. However, it was not detected in stem tissue, protein extracts from the pollen, or unprocessed honey. Maximum expression was 0.001% of total plant protein.

- The expressed PAT enzyme was compared to the bacterial protein: molecular weights were similar, indicating that the protein had not been glycosylated nor had it undergone post transcriptional modifications. Studies showed that the enzyme was inactivated within one minute when subjected to typical mammalian stomach conditions and was inactivated during processing of canola seed into feed ingredients.

- The gene nucleotide sequence and the enzyme amino acid sequence were provided. The nucleotide sequence showed no significant homology the toxins or allergens entered in to GENEBANK DNA database.

2. Kanamycin Resistance:

- Kanamycin is an aminoglycosidic antibiotic that binds to bacterial ribosomes thus disrupting normal protein synthesis and killing the bacterial cell.

- The kanamycin-resistance gene codes for an enzyme that prevents kanamycin from binding to ribosomes, thereby rendering the cells resistant. The exact nature of the enzyme is considered Confidential Business Information by AgrEvo. The source of the gene was described, and the full nucleotide sequence was provided.

- The gene is linked to a weak constitutive promoter; expression was consistently stronger in root tissue, but was also observed in buds, leaves, and crude seed samples. The enzyme was not detected in unprocessed honey or pollen samples and was inactivated during processing of canola seed into feed ingredients.

- The expressed enzyme was compared to the bacterial protein: molecular weights were similar, indicating that the protein had not been glycosylated nor had it undergone post-transcriptional modifications.

- The nucleotide sequence showed no significant homology with the toxins or allergens entered in the GENEBANK DNA database.

3. Development Method:

- *Brassica napus* cultivar Topas was transformed using a disarmed non-pathogenic *Agrobacterium tumefaciens* vector; the vector contained the T-DNA region of an *Agrobacterium* plasmid from which virulence and plant disease-causing genes were removed, and replaced with genes coding for glufosinate ammonium tolerance and kanamycin resistance. The T-DNA portion of the plasmid is known to insert randomly into the plant's genome and the insertion is usually stable, as was shown to be the case in HCN92.

- The transformant was crossed with *B. napus* line ACSN3, then with AC Excel; HCN92 was derived from a bulk of single F_3 plants selected from the cross.

4. Stable Integration into the Plant's Genome:

- The provided data showed that there was no incorporation of any coding region from outside the T-DNA borders and that gene integration occurred at only one insertion site.

- HCN92 is several generations removed from the original transformant. Comparisons between the original transgenic plant and the HCN92 line show no difference in the presence and expression of both genes, nor in the insertion site.

IV. Assessment Criteria for Environmental Safety

1. Potential of the PNT to become a weed of agriculture or to be invasive of natural habitats

AAFC evaluated data submitted by AgrEvo on the reproductive and survival biology of HCN92, and determined that vegetative vigor, overwintering capacity, flowering period, time to maturity, seed production, and dormancy were within the normal range of expression of characteristics in unmodified *B. napus* counterparts. HCN92 has no specific added genes for cold tolerance or winter hibernation; no overwintered plants were observed by AgrEvo in post-harvest

years of field trials, and the number of volunteers in the year following a field trial were comparable between plots of HCN92 and counterpart *B. napus*. Seed morphology and average seed weight did not change, indicating that seed dispersal potential was not altered.

Based on the submitted data, AAFC has determined that HCN92 did not show any stress adaptation other than its resistance to glufosinate ammonium. Its resistance or susceptibility to major *B. napus* pests and pathogens (e.g., blackleg, sclerotinia, flea beetles) fall within the ranges currently displayed by commercial varieties. Moisture stress had a significant negative effect on both HCN92 and its counterparts.

The biology of *B. napus*, described in Dir94-09, shows that unmodified plants of this species are not invasive of unmanaged habitats in Canada. According to the information provided by AgrEvo, HCN92 was determined not to be different from its counterparts in this respect. Invasiveness was studied in disturbed and undisturbed habitats. Data showed that HCN92 was neither more invasive nor more persistent than commercial counterparts. No competitive advantage was conferred to glufosinate ammonium-tolerant plants, other than that conferred by tolerance to glufosinate ammonium.

Glufosinate ammonium is not used in normal crop rotation cycles, and resistance is therefore not an issue of concern in weed management control. Glufosinate-resistant *B. napus* volunteer plants can easily be managed by mechanical means and other available chemicals used to control *B. napus*.

The above considerations, together with the fact that the novel traits have no intended effect on weediness or invasiveness, led AAFC to conclude that HCN92 has no altered weed or invasiveness potential compared to currently commercialized canola varieties.

NOTE: A longer term concern, if there is general adoption of several different crop and specific herbicide weed management systems, is the potential development of crop volunteers with a combination of novel resistances to different herbicides. This could result in the loss of the use of these herbicides and any of their potential benefits. Therefore, agricultural extension personnel, in both the private and public sectors, should promote careful management practices for growers who use these herbicide tolerant crops, to minimize the development of multiple resistance.

2. Potential for Gene Flow to Wild Relatives Whose Hybrid Offspring May Become More Weedy or More Invasive

Brassica napus plants are known to outcross up to 30% with other plants of the same species, and potentially with plants of the species *B. rapa*, *B. juncea*, *B. carinata*, *B. nigra*, *Diplotaxis muralis*, *Raphanus raphanistrum*, and

Erucastrum gallicum (Dir 94-09). Studies show that introgression of the herbicide tolerance gene is most likely to occur with *B. rapa*, the other major canola species and an occasional weed of cultivated land especially in the eastern provinces of Canada.

If glufosinate ammonium-tolerant individuals arose through interspecific or intergeneric hybridization, the novel traits would confer no competitive advantage to these plants unless challenged by glufosinate ammonium. This would only occur in managed ecosystems where glufosinate ammonium is used for broad spectrum weed control, e.g., in the cultivation of plant cultivars developed to exhibit glufosinate ammonium tolerance and in which glufosinate ammonium is used to control weeds. As with glufosinate ammonium-tolerant *B. napus*, these herbicide tolerant individuals, should they arise, would be easily controlled using mechanical and other available chemical means. Hybrids, if they developed, could potentially result in the loss of glufosinate ammonium as a tool to control these species. This, however, can be avoided by the use of sound crop management practices.

The above considerations led AAFC to conclude that gene flow from HCN92 to canola relatives is possible, but would not result in increased weediness or invasiveness of these relatives.

3. Altered Plant Pest Potential

The intended effects of both novel traits are unrelated to plant pest potential, and *Brassica napus* is not a plant pest in Canada (Dir94-09). In addition, agronomic characteristics, stress adaptation, and qualitative and quantitative composition of HCN92 were shown to be within the range of values displayed by currently commercialized *B. napus* varieties, leading to the conclusion that plant pest potential was not inadvertently altered.

AAFC has therefore determined that HCN92 did not display any altered pest potential.

4. Potential Impact on Non-Target Organisms

Data presenting the effect of plant residue from HCN92 on agronomic performance of succeeding crops were examined by AAFC for wheat, barley, lentils, peas, flax and alfalfa. No significant differences in either plant counts or grain yield between the HCN92 and counterpart canola plots were identified. This is an indirect indication that soil bacteria, involved in maintaining soil fertility, are not negatively affected by HCN92 plant residues.

PAT activity was not detected in pollen grains, neither was it detected in unprocessed honey collected from a bee colony which had foraged in the glufosinate-tolerant *B. napus* line. No negative impact on bees foraging in

HCN92 was observed, including brood development. Both enzymes are rapidly inactivated in mammalian stomach and intestinal fluids by enzymatic degradation and

pH-mediated proteolysis. Neither of the two novel proteins contained potential glycosylation sites nor did they possess proteolytic or heat stability, indicating that neither protein is a likely allergen. A search of the GENEBANK DNA sequence database revealed no significant homology with the toxins or allergens entered in that database.

Based on the above, AAFC has determined that the unconfined release of HCN92 will not result in altered impacts on interacting organisms, including humans, compared with currently commercialized counterparts.

5. Potential Impact on Biodiversity

HCN92 has no novel phenotypic characteristics which would extend its use beyond the current geographic range of canola production in Canada. Since outcross species are only found in disturbed habitats, transfer of novel traits would not impact unmanaged environments. Studies have shown to AAFC that HCN92 is not invasive of natural habitats, and that it is no more competitive than its counterparts, both in natural and managed ecosystems.

AAFC has therefore concluded that the potential impact on biodiversity of HCN92 is equivalent to that of currently commercialized canola lines.

V. Assessment Criteria for Use as Livestock Feed

1. Anti-Nutritional Factors

Ninety-five percent confidence intervals were determined for glucosinolate and erucic acid content of the meal and oil produced from HCN92, grown under a variety of conditions. These confidence intervals demonstrated that the PNT contained levels of these anti-nutritional factors below the prescribed standards for both the meal and oil fractions, i.e., <30 micromoles glucosinolates per gram of dry meal and <2% erucic acid in the oil.

2. Nutritional Composition of PNT

No statistical differences in nutritional composition, i.e., crude protein, crude fat, crude fibre, ash and gross energy content, were noted between the whole seed, processed meal or oil of HCN92 and current commercial canola cultivars. These results collectively demonstrate that the introduction of this construct into *B. napus*, resulting in HCN92, did not likely result in any secondary effects impacting on the composition or nutritional quality of the cultivar. Accordingly, HCN92 was judged to be substantially equivalent to traditional canola varieties in terms of nutritional composition.

VI. Regulatory Decision

Based on the review of data and information submitted by AgrEvo Canada Inc., and through thorough comparisons of HCN92 with unmodified *B. napus* counterparts, AAFC has concluded that neither the novel genes, nor their resulting gene products and associated novel traits, confer any intended or unintended ecological advantage to HCN92. Should these traits be transferred through outcrossing to related plants, these would not result in any ecological advantage.

Based on the review of submitted data and information, AAFC has concluded that the novel genes and their corresponding traits do not in themselves raise any concerns regarding the safety or nutritional composition of this line. Canola oil and meal are currently described in Schedule IV of the *Feeds Regulations* and are therefore approved for use in livestock feeds in Canada. As HCN92 has been assessed and found to be substantially equivalent, HCN92 and its by-products are considered to meet the present definitions and are approved for use as livestock feed ingredients in Canada.

Unconfined release into the environment, including feed use of HCN92, and other *B. napus* lines derived from it, but without the introduction of any other novel trait, is therefore considered safe.